SURVIVAL AND REPRODUCTION TEST WITH CLADOCERAN
(*Ceriodaphnia dubia*)

1. TEST OBJECTIVE

To assess the toxicity of a test material to *Ceriodaphnia dubia* and determine the effects on reproductive potential and survival of test organisms compared to controls.

2. TEST ARTICLE

2.1 Description/Identification

Unless otherwise specified, the test material is supplied by the client. Adequate chemical specifications with special reference to hazardous properties and storage conditions are also supplied by the client.

2.2 Methods of Synthesis

In most cases, the test article is an effluent sample. Information on the methods of synthesis, stability, and composition or other characteristics which define the test article are on file with the client.

3. EXPERIMENTAL DESIGN

3.1 Test Organisms

3.1.1 Species

The test species is the water flea, *Ceriodaphnia dubia*.

3.1.2 Source

C. dubia used for toxicity tests are obtained from stock cultures maintained at EA's Culture Facility.

3.1.3 Culturing and Holding Conditions

C. dubia cultures are maintained at 25±2°C and a 16-hour light, 8-hour dark photoperiod cycle in an environmentally controlled laboratory. Test organisms are maintained in 18.9-L all glass

aquaria (as backup cultures) or 1-L culture bowls, or individually in 30-ml plastic portion cups in brood boards and fed algae and a trout chow/yeast/cerophyll suspension (US EPA 1994). New cultures are initiated on a routine basis to ensure healthy, productive populations. Organisms from cultures producing ephippia are not used for toxicity tests. Certain regulatory or project specific objectives may require organism acclimation to the dilution water when it is different from the holding/culture water.

3.1.4 Age of Test Organisms at Test Initiation

Neonates of known age (i.e., less than 24 hours old) are obtained for testing from the individually cultured females in the brood board system. On the day before or the day of the test, neonates are segregated from the parent organisms. All organisms used for testing are released within one 8-hour period and are taken from broods of eight or more.

3.2 Dilution Water

Twenty percent dilute mineral water (US EPA 1994) is used for culturing and testing. Alternatively, moderately hard reconstituted water is prepared using MILLIPORE MILLI-Q^{R} or equivalent deionized water with addition of reagent grade chemicals. Receiving water can be used as dilution water if specified by the client.

3.3 Test Concentration Series

The test concentration series consists of a minimum of five dilutions (e.g., 6.25, 12.5, 25, 50, and 100 percent effluent plus a control) and may be determined from a prior screening of the test material. Rangefinding assays utilize more widely spaced test concentrations and a control. Ambient water or effluent samples may also be evaluated as single concentrations and compared to a control.

3.4 Test Concentration Preparation

Test concentrations are prepared in 250-ml graduated cylinders, and 15 ml of test solution are delivered to each individual test chamber.

3.5 Test Vessels and Test Volume

Test vessels are 30-ml portion cups or beakers; the final test volume is 15 ml.

3.6 Test Organism Number

Tests are conducted using ten replicates per concentration, with one organism per container. Neonates are randomly assigned to each replicate test container. More replicates can be added, if appropriate.

3.7 Test Environment

The test vessels are maintained in an environmentally controlled laboratory with a 16-hour light, 8-hour dark photoperiod. Temperature within the environmental room is monitored continuously using temperature recorders and test vessels are maintained at 25±1°C (unless a different project-specific temperature is required).

3.8 Analysis of Test Concentrations for Test Article

If required, test solutions may be analyzed for verification of chemical concentrations. The analytical method and number of analyses are determined after consultation with the client. When chemical analyses are necessary, both nominal and actual measured test concentrations are reported.

3.9 Test Observations

Each test day, test organisms are observed to record the number of surviving organisms and the number of neonates produced. Dead organisms are removed when observed. The study terminates after 60 percent of the control females have produced three broods with a minimum of 15 neonates per surviving female. However, test duration will not exceed 8 days of exposure to the test material.

Each effluent or receiving water sample received is analyzed for temperature, conductivity, alkalinity, hardness, and total residual chlorine. Aliquots of effluent and receiving water may be gently aerated (100 bubbles/min) prior to test initiation if dissolved oxygen is less than 4 mg/L or greater than 100 percent saturation. Water quality measurements recorded daily on old and new test solutions include dissolved oxygen, pH, temperature, and conductivity from a minimum of one replicate of every concentration. Analytical determinations are conducted according to APHA et al. (1995) and US EPA (1979).

3.10 Solution Renewal

The test solutions are renewed daily. New solutions are prepared on the day of renewal. After the new solutions have reached test temperature, the test organisms are transferred from the old test vessels to the new test vessels using a pipet or glass tube. The offspring are counted and the number is recorded. Caution is given not to stress the test organisms during transfer. After

neonates are counted and water quality measurements (temperature, pH, dissolved oxygen, and conductivity) are completed, the old solution is discarded.

3.11 Data Analysis

Statistical analyses are performed on percent survival and young production. Fisher's Exact Test is used to determine statistical significance of the survival data. Analysis of variance (ANOVA) and either Bonferroni's T-test or Dunnett's Mean Comparison test are used to analyze the young production data for significance of effects. Depending on the distributional characteristics of the data generated, it may be necessary to use Steele's Many-One Rank Test or the Wilcoxon Rank Sum Test instead (US EPA 1994). The Shapiro-Wilks test (for datasets with ≤50 datapoints) or the Chi-Square test is used to test for normality of the reproduction data. Bartlett□s test is used to test for homogeneity of variance of the reproduction data. If requested before the initiation of the study, the young production data will be analyzed using EPA's ICp program to determine an IC50 and/or IC25. Although not standard practice, an LC50 may be calculated using the probit, moving average, and binomial methods as described by Stephan (1977). Depending on the nature of the data other methods may be used, including the probit approximation method of Litchfield and Wilcoxon (1949), SAS probit analysis (SAS Institute 1985) or graphical interpolation using the log concentration vs. percent lethality as described by APHA et al. (1995). The methods used are specified in the final report.

3.12 Test Acceptability

An individual test may be conditionally acceptable if temperature, dissolved oxygen, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests.

4. FINAL REPORT

The final report is prepared to contain, at a minimum, the following information:

- □ Objectives and procedures stated in the approved protocol, including any changes made to the original protocol
- □ Identity of the test article(s) by name or code number and their strength (i.e., quality/purity), and a description of any pretreatment
- □ Source of the dilution water, its chemical characteristics, and a description of any pretreatment
- □ Test concentration series used and duration of the assay

- Water quality characteristics (pH, dissolved oxygen, temperature, etc.) of dilution water and selected test concentrations during testing
- Any unforeseen circumstances that may have affected the quality or integrity of the study
- Signature of the project manager, senior technical reviewer, and quality control officer authorizing release of the report
- Location of all archived data and the original copy of the final report at EA

Items of data to be included in the report consist of experimental design and test performance, effects on general appearance of test organisms (if applicable), morbidity and mortality, presentation of water quality characteristics, survival and reproduction data.

5. QUALITY ASSURANCE

5.1 Amendments to Protocol

Amendments to the authorized protocol established by EA or by the client are made only after proper authorization. Such authorization is achieved by completion of the Protocol Amendment Form by EA after consultation with the client.

5.2 Standard Operating Procedures

Unless otherwise specified, all procedures given in the protocol are subject to detailed Standard Operating Procedures (SOPs) which are contained in the SOP manuals of the participating departments. These SOPs and protocols generally follow the types of requirements outlined in the U.S. EPA's Good Laboratory Practice Standards (GLPs)
(US EPA 1989).

5.3 Reference Toxicant

A reference toxicant test, utilizing sodium dodecyl sulfate (SDS), cadmium chloride, NaCl, or another appropriate chemical is used as an internal quality check of the sensitivity of the test organisms. Testing is conducted at least once monthly on organisms that are cultured in-house.

The results of each test are compared with historical, species-specific toxicological information from reference toxicant tests performed at EA, to determine if the results are within acceptable limits. Limits are established using the control charts outlined in US EPA (1994).

5.4 Quality Assurance Evaluation

Studies conducted under this protocol may be subject to internal audit by EA's Quality Assurance Unit. A quality control officer is responsible for monitoring each study to assure the client that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with EA's QC program and, if applicable, EPA's GLPs.

5.5 Inspection by Regulatory Authorities

In the event of an inspection of EA by an outside authority during the course of the study, the client whose study is being inspected will be consulted before examiners are permitted access to any of the project records or the experimental areas.

5.6 Archives

Copies of project-specific records shall be transferred to the client promptly after the project is completed or as negotiated and budgeted. Original primary data are retained at EA for 5 years. Primary data include chain-of-custody records, laboratory data sheets, records, memoranda, notes, photographs, microfilm, and computer printouts that are a result of the original observations and activities of the study and which are necessary for the reconstruction and evaluation of the study report.

5.7 Location

Studies are conducted at the Ecotoxicology Laboratory of EA Engineering, Science, and Technology, Inc. at the Loveton Office in Sparks, Maryland.

6. SPECIFICATIONS OF THE *Ceriodaphnia dubia* SURVIVAL AND REPRODUCTIVE POTENTIAL TEST

6.1 Basic References

American Public Health Association (APHA) American Water Works Association, Water

Environment Federation. 1995. Standard Methods for Examination of Water and Wastewater, 19th or most recent version. APHA, Washington, D.C.

EA. 1996. Quality Control and Standard Operating Procedures Manual for EA's Ecotoxicology Laboratory. Fifth Revision. EA Manual ATS-102. Internal document prepared by EA's Ecotoxicology Laboratory, EA Engineering, Science, and Technology, Inc., Sparks, Maryland.

Litchfield, J.T., and F. Wilcoxon. 1949. A simplified method of Evaluating Dose/Effect Experiments. J. Pharmacol. Exp. Ther. 96:99-113.

SAS Institute Inc. SAS Users Guide: Statistics, Version 5 Edition. Cary, NC:SAS Institute Inc., 1985.

Stephan, C.E. 1977. Methods of Calculating an LC50 in Aquatic Toxicology and Hazard Evaluation (F.L. Mayer and J.L. Hamlink, Eds.), pp. 65-84. ASTM STP 634, ASTM, Philadelphia, Pennsylvania.

US EPA. 1979. Methods for Chemical Analysis of Water and Wastes. EPA/600/4-79/020. U.S. Environmental Protection Agency, Washington, D.C.

US EPA. 1989. Toxic Substances Control Act (TSCA); Good Laboratory Practice Standards. Title 40 CFR Part 792. Fed. Regist. 54(158): 34034-34074.

US EPA. 1989. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Second Edition. EPA/600/4-89/001. U.S. Environmental Protection Agency, Cincinnati, Ohio.

US EPA. 1994. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Third Edition. EPA/600/4-91/002. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

6.2 Test Specifications

Test organism:	Water flea (*Ceriodaphnia dubia*)
Temperature:	Target: 25±1°C
Age:	<24 hours and all released within an 8-hour window

Aeration:	None
Light quality:	Wide-spectrum fluorescent light
Light intensity:	50-100 f.c.
Photoperiod:	16-hour light, 8-hour dark
Dilution water:	20 percent dilute mineral water, dechlorinated municipal tap water, reconstituted fresh water, or appropriate receiving water
Test containers:	30-ml plastic portion cups or beakers
Test volume:	15 ml per replicate
No. of concentrations:	Definitive assay - Minimum of five test concentrations and a control Screening assay - Single test concentration and a control
No. of replicates:	10
No. organisms per replicate:	1
Feeding regime:	200 μl of 5 g/L food solution (YCT and algae mixed) daily in each replicate (according to US EPA 1994)
Test type and duration:	Static renewal with daily replacement of test solutions. The test duration is determined when 60 percent of the control females have three broods; however, not exceeding 8 days.
Endpoints:	Survival of females used to initate test and number of young produced per female over the exposure period

Test acceptability:	80 percent or greater survival and an average of 15 or more young/surviving female in the control solution